attenuated the lifespan of Ant1-deficient mice. Our results prove mtDNA dictates the penetrance of age-related cardiomyopathy and mammalian lifespan. Therefore, therapeutics that most effectively preserve mitochondrial DNA and bioenergetics will provide the most promise for healthy aging.

3066-Pos Board B496

Activation of the Mitochondrial Permeability Transition Pore Leads to the Increase in Amount of C-Subunit of ATP Synthase Associated with Channel-Forming Complex of Polyhydroxybutyrate and Inorganic Polyphosphate

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Low permeability of the mitochondrial inner membrane is critical for maintaining the mitochondrial electrochemical potential - the driving force for ATP production. Acute stress conditions, lead to the increase in the mitochondrial inner membrane permeability due to the opening of the permeability transition pore (PTP). PTP allows free movement of ions and small molecules leading to mitochondrial depolarization, ATP depletion and cell death. Recent studies suggest that C-subunit of the mitochondrial ATP synthase plays a central role in PTP. Previous work in our laboratory showed that mitochondria contain non-protein complex composed of polyhydroxybutyrate, inorganic polyphosphate and calcium that forms an ion channel with properties resembling PTP. Here we explore the possibility of interactions between these non-protein components and C-subunit during the induction of PTP.

To induce PTP,isolated energized mitochondria were treated with calcium. Control mitochondria were treated with calcium either in the presence of ruthenium red, inhibitor of calcium uptake or Cyclosporin A, inhibitor of PTP. This was followed by a water-free chloroform extraction of channel forming fraction of PTP. Components of the extract were analyzed using immunoblot analysis. We found significantly increased amount of C-subunit associated with channel forming fraction extracted from mitochondria with activated PTP. In contrast, C-subunit was not detectable in the extract when Ruthenium Red was present and significantly decreased in the presence of Cyclosporin A or in the absence of calcium.

These results show that C-subunit is likely an interacting partner of the pore-forming complex of polyphosphate, calcium and polyhydroxybutyrate. We hypothesize that fully functional PTP requires calcium-induced formation of the pore made of both the complexed polymers and the c-subunit of ATP synthase

3067-Pos Board B497

Small-Molecule PKD Inhibitor Prevents Mitochondrial Fragmentation and Dysfunction during Gq-Protein Coupled Receptor Stimulation in Cardiac Cells

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Regulation of mitochondrial morphology and dynamics is crucial for the maintenance of various cellular functions in cardiomyocytes. Abnormal mitochondrial morphologies concomitant with mitochondrial dysfunction are frequently observed both in human heart failure (HF) and in animal HF models. However, it is still unclear which cardiac signaling pathways regulate mitochondrial morphology and function under pathophysiological conditions. Recent reports suggest that G_q-protein coupled receptor (G_qPCR) signaling pathways are critical for the development and progression of HF. Therefore, we hypothesize that G_qPCR stimulation induces mitochondrial fragmentation and dysfunction, which initiates cardiomyocyte death. We found that protein kinase D (PKD) activated by G_qPCR signaling was translocated to outer mitochondrial membrane (OMM) observed by Western blot analysis of cytosolic and mitochondria-enriched fractionated proteins and by live cell imaging of fluorescence resonance energy transfer (FRET). We also found that G_qPCR-mediated PKD activation induced mitochondrial fragmentation, leading to increased reactive oxygen species (ROS) generation as well as increased mitochondrial permeability transition pore (mPTP) opening, which initiates apoptotic signaling activation and cardiomyocyte death. These morphological and functional changes in cardiac mitochondria were mediated via PKD-dependent phosphorylation of mitochondrial fission protein, Dynamin-Like Protein 1 (DLP1) at S637. Moreover, pretreatment with a novel potent PKD inhibitor CRT0066101 effectively inhibited G_qPCR-mediated PKD translocation to OMM, DLP1 phosphorylation at S637, mitochondrial fragmentation, ROS generation and mPTP activation. In conclusion, we demonstrate that G_qPCR stimulation induces mitochondrial fragmentation and dysfunction through PKD-dependent phosphorylation of DLP1 at S637, which likely contributes to cardiomyocyte injury. Thus, small-molecule PKD inhibitor may become a novel and potent therapeutic for preventing cardiac cell injury and death during HF.

3068-Pos Board B498

EPR Data Support the Existence of a Symmetric BH3-in-Groove Homodimer in Oligomeric BAK

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The BAX or BAK oligomeric pore formation in the mitochondrial outer membrane is a critical step in apoptosis, yet their structures are not clearly understood. Czabotar *et al.* (Cell 2013, **152**, 519) reported a crystal structure of a water-soluble tetramer of the BAX fragment (helices α2-α5) fused to the green-fluorescent protein (GFP), in which the α2-α3 extended helices and the α5 helix, respectively, were juxtaposed to each other, in an anti-parallel orientation, forming a symmetric "BH3-in-groove homodimer (BGH)." We have constructed a GFP-BAK fusion protein using the α2-α5 helices of mouse BAK, designated as GFP-BAKα2-α5, which also forms a soluble tetramer. To determine whether the BGH exists in the BAK oligomers in the membrane or not, we spin labeled the C-terminal hexahistidine-tagged soluble form of mouse BAK (helices α1-α8) at residues 84, 122, 128 and 135 and the corresponding residues in GFP-BAKα2-α5. We then compared the continuous wave (CW) EPR spectra of the spin-labeled residues from the tetrameric GFP-BAKα2-α5 with those from the oligomeric BAK in membrane. Spin labeled residue 122R1, located in the loop interconnecting helices α4 and α5 in the homology model of the GFP-BAKα2-α5 tetramer, displayed a mobile lineshape. The corresponding residue in the oligomeric BAK also had a remarkably similar lineshape, indicating that the two residues are in similar structural environments. Residues 84R1, 128R1 and 135R1, located at the anti-parallel helical interfaces in the BGH also had remarkably similar immobile lineshapes both in the GFP-BAKα2-α5 tetramer and in the oligomeric BAK in membrane, further strengthening the above conclusion. The intra-dimer distances between 84R1 spin label pairs in the GFP-BAKα2-α5 and the oligomeric BAK, determined by the double electron electron resonance (DEER) method, also support this interpretation.

3069-Pos Board B499

Modulation of Membrane Interactions of Anti-Apoptotic Regulator Bcl-xL by Lipids

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The Bcl-2 family of proteins (e.g., pro-apoptotic Bax and anti-apoptotic Bcl-xL) regulates the mitochondrial outer membrane permeabilization during the early stages of apoptosis. The prevalent Embedded Together Model of Bcl-2 action suggests that the membrane environment is critical for their proper functional interactions, consistent with the increasing evidence of lipids being involved in the regulation of apoptotic response. In this study, we apply a collection of fluorescence-based methods to investigate the effect of various lipids on the pH-triggered membrane interactions of Bcl-xL. The initial membrane association was studied using a FRET assay with donor-labeled Bcl-xL and acceptor-labeled vesicles, while the insertion/refolding of Bcl-xL into the membrane was monitored using the environment-sensitive probe NBD selectively attached in the middle of hydrophobic helix α6. Our results demonstrate that the lipid composition affects the pH-dependence of both initial membrane association and subsequent insertion/refolding of Bcl-xL. We found that a linear correlation exists between the membrane surface potential created by anionic lipids and the pKa of membrane binding, suggesting that the initial step is controlled by an electrostatic mechanism. The effect of lipids on the membrane insertion/refolding step is more complex and appears to be influenced by the size of the lipid headgroup. The kinetics of both the membrane association and membrane insertion/refolding is affected by the presence of non-bilayer forming lipids commonly found in mitochondria. While the presence of phosphatidylethanolamine accelerated the process, addition of lysophosphatidylcholine had the opposite effect, suggesting that mechanical properties of the bilayer also play a role. Taken together our results indicate that lipids can modulate the membrane interactions of Bcl-xL in multiple ways, providing an additional regulatory mechanism that ensures proper control of a complex cascade of apoptotic reactions leading to cell death or survival. NIHGM-069783, Fulbright-CONICYT, BRTP.

3070-Pos Board B500

Binding of Pro-Apoptotic Protein Bax to Cytoprotective UDCA and TUDCA

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Hydrophobic bile acids, such as deoxycholic acid (DCA), strongly induce apoptosis in both hepatic and non-hepatic cells while hydrophilic bile acids,

like ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid (TUDCA), are cytoprotective and inhibit cell death. The mechanisms associated with these distinct effects are not entirely clear. However, the effect of hydrophilic bile acids seems to be related with the blockage of a series of processes that converge on mitochondrial damage. Bax is a pro-apoptotic protein that belongs to the superfamily of the Bcl-2 proteins and is involved in mitochondrial pore formation. Submicellar concentrations of cytoprotective bile acids have been shown to modulate Bax concentration in mitochondria, suggesting that these molecules may interact directly with the protein. In this study, our objective was to evaluate the affinity of bile acids to recombinant Bax protein, making use of fluorescence spectroscopy (FRET and fluorescence anisotropy), as well as Fluorescence Correlation Spectroscopy (FCS). Our results show that the cytoprotective bile acids UDCA and TUDCA associate with recombinant Bax protein with high affinity, while the cytotoxic bile acid DCA only seems to be able to adsorb to the protein with much lower affinity. Notably, the binding site for UDCA seems to be located in a hydrophobic pocket of the protein. This interaction could be responsible for the disruption of Bax translocation to the mitochondrial outer membrane in the presence of UDCA and/or TUDCA.
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3071-Pos Board B501

MAC Inhibitors Neutralize the Pro-Apoptotic Effects of Tbid

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Since our initial characterization of the iMACs, different di-bromocarbazole derivatives with anti-apoptotic function have been developed and tested in several mouse models of brain injury and neurodegeneration [13-21]. Owing to the increased therapeutic potential of anti-apoptotic di-bromocarbazole derivatives, we sought to expand our knowledge of the mechanism of action of these small molecule inhibitors. We investigated the kinetics of MAC inhibition in mitochondria from wild type, Bak, and Bax knockout cell lines using patch clamp electrophysiology, fluorescence microscopy, ELISA, and quantitative western blot analyses. Our results show that iMACs work through at least two mechanisms: 1) by blocking relocation of the cytoplasmic Bax protein to mitochondria and 2) by disassembling Bax oligomers in the outer membrane. A comparison of the inhibitory effects over channel conductance and cytochrome c release suggests that the iMACs interacted with both Bax and Bak with similar kinetics. Interestingly, wild type mitochondria were more susceptible to inhibition than the Bak or Bax knockouts. A quantitative western blot analysis showed that wild type mitochondria had lower steady state levels of Bak, which suggests an uneven stoichiometry of the MAC components.

3072-Pos Board B502

Tyrosine Phosphorylation of Mitochondrial Ca^{2+} Uniporter Regulates Mitochondrial Ca^{2+} Uptake

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Mitochondrial Ca^{2+} has a critical role for balancing cell survival and death. Ca^{2+} influx into mitochondrial matrix is mediated primarily by the mitochondrial calcium uniporter (MCU). However, the signaling pathways that regulate MCU channel functions via post-translational modifications of MCU are completely unknown. Here we show that adrenergic signaling induces MCU tyrosine phosphorylation and accelerates mitochondrial Ca^{2+} uptake in cardiac cells. Adrenergic signaling induces activation of proline-rich tyrosine kinase 2 (Pyk2) and translocation into the mitochondrial matrix; enhancing the interaction between Pyk2 and MCU, which subsequently accelerates mitochondrial Ca^{2+} uptake via Pyk2-dependent MCU tyrosine phosphorylation. MCU contains 15 tyrosine residues (5 in the N-terminus, 0 in the pore-forming region, 4 in transmembrane domains and 6 in the C-terminus), which are conserved across all eukaryotic species. Among them, only 3 of these tyrosine residues (Y157 at N-terminus, Y288, and Y316 at C-terminus in mouse MCU) remained as potential phosphorylation candidate sites for protein tyrosine kinases using phosphorylation prediction programs. We mutated these tyrosine residues to phenylalanine and generated non-phosphorylation mimetic MCU mutants (MCU-YFs). We confirmed that only two tyrosine sites were phosphorylated in response to adrenergic stimulation *in situ* using cell lines stably expressing MCU-YFs. In addition, overexpression of these MCU-YFs failed to increase mitochondrial Ca^{2+} uptake in response to cytosolic Ca^{2+} elevation by thapsigargin, whereas wild-type MCU transfection dramatically accelerates mitochondrial Ca^{2+} uptake compared to non-transfected cells. In summary, MCU contains Pyk2-specific phosphorylation site(s) and Pyk2-dependent tyrosine phosphorylation of MCU can modulate its channel functions and regulate mitochondrial Ca^{2+} uptake.

3073-Pos Board B503

Cardioprotective Roles of Neuronal Ca^{2+} Sensor-1 during Stress

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Dysregulation of Ca^{2+} homeostasis in cardiomyocytes often results in heart failure. Identifying molecular targets that regulate cardiomyocyte survival is of therapeutic importance. Neuronal Ca^{2+}-sensor-1 (NCS-1) is an EF-hand Ca^{2+}-binding protein, which is important for excitable cell functions. We previously found that NCS-1-deficient *($Ncs1^{-/-}$)* mice had excess neonatal mortality (*Circ. Res. 2011*). The aim of the present study is to examine whether NCS-1 plays beneficial roles in cardiac survival during stress and the possible mechanisms underlying these effects. Neonatal mouse ventricular myocytes or whole hearts from wild-type (WT) and *$Ncs1^{-/-}$* mice were subjected to stressors, and the resistance to stress was evaluated. *$Ncs1^{-/-}$* mouse hearts were more susceptible to stress induced by oxidative impairment and ischemia-reperfusion injury. Stress-induced activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling, a major survival pathway, was substantially reduced in the *$Ncs1^{-/-}$* group, and overexpression of NCS-1 or the constitutive active form of Akt increased the survivalrate of *$Ncs1^{-/-}$* myocytes. Cellular ATP levels, as well as mitochondrial respiration rates (both basal and maximal O_2 consumption) were significantly depressed in *$Ncs1^{-/-}$* myocytes; especially with oxidative stress. Furthermore, intracellular Ca^{2+} handling was more easily dysregulated in stressed *$Ncs1^{-/-}$* myocytes than WT myocytes. Since NCS-1 levels were increased by stress, the data suggest that NCS-1 is a survival-promoting factor, which is upregulated by stress stimuli. Interestingly, however,supra-physiological NCS-1 expression was toxic to cells. Taken together, our data suggest that moderate NCS-1 expression during stress promotes cardiomyocyte survival by maintaining proper Ca^{2+} handling, which is required for activation of Akt survival pathways and mitochondrial function.

3074-Pos Board B504

Initiation of Electron Transport Activity and a Decrease of Oxidative Stress Occur Simultaneously during Embryonic Heart Development

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Mitochondria in early embryonic hearts are not thought to produce ATP, yet they do produce reactive oxygen species (ROS) that regulate myocyte differentiation. To assess changes in ATP and ROS generation in the developing heart, we measured mitochondrial oxygen consumption, the activity of the complexes (Cx) 1 and 2 of the electron transport chain (ETC), ETC supercomplex assembly, and ROS in embryonic mouse hearts. At embryonic day (E) 9.5, mitochondrial ETC activity and oxidative phosphorylation (OXPHOS) are not coupled, even though the ETC complexes are present. We show that Cx-1 is able to accept electrons from the Krebs cycle, but enzyme assays that specifically measure electron flow to ubiquinone or Cx-3 show no activity at this early embryonic stage. At E11.5, mitochondria appear functionally more mature; ETC activity and OXPHOS are coupled and respond to ETC inhibitors. In addition, the assembly of highly efficient respiratory supercomplexes containing Cx −1, −3, and −4, ubiquinone, and cytochrome *c* begins at E11.5, the exact time when Cx-1 becomes functional activated. At E13.5, ETC activity and OXPHOS of embryonic heart mitochondria are indistinguishable from adult mitochondria. In contrast, generation of reactive oxygen species (ROS), as measured with Amplex Red, is high at E9.5 and drops significantly by E11.5, coinciding with activation of the ETC. In summary, our data suggest that between E9.5 and E11.5 dramatic changes occur in the mitochondria of the embryonic heart, which result in a decrease of ROS generation and an increase in OXPHOS due to the activation of Cx-1 and the formation of supercomplexes.

3075-Pos Board B505

The Stoichiometry between MICU1 and MCU Determines the Different Mitochondrial Ca^{2+} Uptake Phenotypes in Heart and Liver

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Mitochondrial Ca^{2+} uptake is central to oxidative metabolism and cell death signaling. The first clues to its molecular mechanism have emerged from the recent identification of the mitochondrial Ca^{2+} uniporter's pore forming protein (MCU) as well as its regulators. Among the regulators, MICU1 shows striking